

Fusogenic domain and lysines in saposin C<sup>☆</sup>

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## Abstract

Saposin C, a sphingolipid activator protein with fusogenic activity, interacts specifically with the membrane containing negatively charged, unsaturated phospholipids. The kinetics and mechanism of saposin C-induced membrane fusion were previously investigated using acidic phospholipid liposomes. A hypothetic clip-on model for such a fusion process was illustrated by the ionic binding between saposin C and lipids, as well as the inter-saposin C hydrophobic interaction. Here, we report the location of the fusogenic domain in a linear sequence at the amino-terminal half of saposin C. This domain consisted of the first and second helical sequences. Selected positively charged lysines in the fusogenic domain were mutated to study the roles of basic residues in the saposin C-induced vesicle fusion. Based on the results, Lys13 and Lys17 were critical for the fusogenic activity, but had no effect on the enzymatic activation of acid  $\beta$ -glucosidase (GCase). These results clearly indicate the segregation of the fusion and activation function into two different regions of saposin C. Interestingly, all the Lys mutant saposin Cs anchored on the acidic phospholipid membrane. Our data suggest that saposin C's fusogenic and activation functions have different requirements for the orientation and insertion manners of helical peptides in membranes.

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Saposins are a family of heat-stable, protease-resistant glycoproteins which consist of multi-amphipathic helices in a bundle structure [1–3]. Four members of the saposin family, namely saposins A, B, C, and D, are encoded by a single prosaposin gene [4–7]. These saposins, with a high sequence similarity (up to 60%), share several common structural features, such as one glycosylation site with conserved placement in the N-terminal half of the sequence and three disulfide bonds formed with a strictly conserved placement of six cysteines. Glycosylation is not essential to their activities [8,9], but may be needed for normal intracellular stability [8,10]. The amino acid sequences and structures of saposins B and C are illustrated in Fig. 1. The three disulfide bonds and the location of the multi-amphipathic helices are indicated. The 3-D structure of saposin B was resolved by X-ray crystallization [2]. The disulfide bonds and helical bundle structure confer their great thermostability and intracellular stability [3,11,12].

The saposin family has been subjected to numerous functional studies. The functionalities of saposins have been diversified in various biological processing. Saposins B and C are required for *in vivo* activities of aryl-sulfatase A and acid  $\beta$ -glucosidase (GCase), respectively [13]. In addition to their stimulation effects on various lysosomal hydrolyases, saposins have membrane binding and lipid transport properties [14]. Lipid membrane destabilization by saposins at acidic pH leads to a lytic reaction on vesicles [15,16]. Recently, saposins have been subjected to extensive studies of their interactions and impacts on various model membranes. Saposins promote the reorganization of phospholipid bilayers, such as lipid microdomain formation and thickness reduction on bilayers [17,18]. Saposins have been shown to anchor onto lipid membranes by embedding into the outer leaflet of the bilayer [19]. Two helices, H-1 [saposin C (4–20)] and H-5 [saposin C (67–80)], participate in this insertion process (Fig. 1). Although saposins have the general property of lipid membrane binding activity, saposin C showed a different interaction mode on the membrane [19]. More specifically, the liposomal membrane fusion at low pH was induced by saposin C at nanomolar concentrations unlike other saposins [20].

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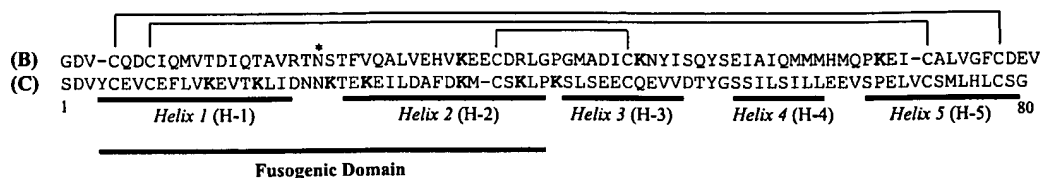


Fig. 1. Amino acid sequences of human saposins B and C. Saposins B and C are small glycoproteins with a conserved N-glycosylation consensus sequence (asterisk) and six cysteines. The three disulfide bonds and the locations of the predicted  $\alpha$ -helices are indicated. The lysines residues are shown in boldface. The fusogenic domain in the saposin C sequence is indicated.

Saposin C is fusogenic, since it promotes the biological membrane fusion at acidic pH. The fusogenic activity of saposin C is influenced by the composition of the membranes. Saposin C-induced membrane fusion with acidic lipid containing vesicles has been observed by electron microscopy, a particle size analyzer, and fluorescence spectroscopy [15,20]. The kinetics and mechanisms of saposin C-induced membrane fusion are characterized using liposomal vesicles [20]. Kinetic studies show that the initial fusion burst by saposin C is completed in the timeframe of a few seconds to minutes based upon the phospholipid content. Fusogenic activity of saposin C is dependent upon unsaturated, anionic phospholipids, and acidic pH. At least two events were found in saposin C-membrane association: membrane anchoring of the terminal helices (H-1 and H-5) and reorientation of its central helical region (H-2, H-3, and H-4). Importantly, saposin-lipid and saposin-saposin interactions are needed for the fusion process [19]. Thus, the proper membrane insertion and helical orientation of saposin C may be important for its specific fusogenic activity. In addition, structural changes of the membrane on a nano-scale range are induced by saposin C, including striking topographic changes and development of patch-like features [17,18]. The helical peptides in saposin C, H-1, and H-2 [saposin C (24–40)] showed dominant effects on membrane reorganization. Since the basic amino acid residues are located in sequence 1–41 of saposin C, the region of H-1 and H-2 helices may play a critical role in the interaction of saposin C with acidic lipid membranes. Such saposin C-membrane association is likely to provide a molecular basis for membrane fusion. In the present study, we report that the linear amino acid sequence at the  $\text{NH}_3$ -terminal half of saposin C had a potent fusogenic activity towards acidic phospholipid vesicles. Selected lysine residues in this domain are necessary for fusogenic activity, but are not required for enzymatic activation of GCase.

## Materials and methods

### Materials

The following materials were from commercial sources: Quick change site-directed mutagenesis kit (Strata-

gene, La Jolla, CA); magic polymerase chain reaction prep kits (Promega, Madison, WI); pET21a(+) DNA vector, *Escherichia coli* host strain [BL21(DE3)], and His-Bind resin (Novagen, Madison, WI); octadecyl rhodamine B chloride (R18) (Molecular Probes, Eugene, OR); synthesized human saposin C peptides (Genemed Synthesis, San Francisco, CA);  $\text{C}_4$  reverse-phase HPLC<sup>1</sup> column (Alltech Association, Deerfield, IL); PD-10 (Sephadex G-25M) columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden); ceredase (Genzyme, Boston, MA); brain phosphatidylserine (BPS), oleoyl lysobisphosphatidic acid (*S,R* isomer)*sn*-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-*sn*-3'-(1'-oleoyl-2'-hydroxy)-glycerol (dioleoyl-LBPA), and egg phosphatidylcholine (EPC) as stock solutions in chloroform (Avanti Polar Lipids, Alabaster, AL). Anionic lipids were sodium salts. All other chemicals were of reagent grade or better.

### Protein preparation

All recombinant saposins were overexpressed in *E. coli* cells by using an IPTG-induction pET system [9]. The amino acid sequences of human saposins B and C are in Fig. 1. The mature  $\text{NH}_2$ -terminal amino acid from the natural human saposins was assigned as 1 and used to identify the amino acids in the fragments. The cDNAs for all mutant saposins were generated using the QuickChange site-directed mutagenesis kit and validated by complete DNA sequencing. All expressed proteins contained a His-tag and were purified on a nickel column and with  $\text{C}_4$  reverse-phase HPLC chromatography using a linear (0–100%) gradient of acetonitrile in 0.1% trifluoroacetic acid. The major protein peak was collected and lyophilized. The protein concentrations were determined as previously described [9].

### Vesicle preparation

Large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs) were prepared by bath and tip

<sup>1</sup> Abbreviations used: HPLC, high performance liquid chromatography; BPS, brain phosphatidylserine; EPC, egg phosphatidylcholine; dioleoyl-LBPA, oleoyl lysobisphosphatidic acid (*S,R* isomer)*sn*-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-*sn*-3'-(1'-oleoyl-2'-hydroxy)-glycerol; R18, octadecyl rhodamine B; SDP, size distribution process; CD, circular dichroism; GCase, acid  $\beta$ -glucosidase.

sonications, respectively [19,20]. Phospholipids (total lipids = 20  $\mu$ mol) in chloroform were dried under  $N_2$  and vacuum. The lipid films were suspended in 0.1 M citric acid/0.2 M phosphate (pH 4.7) and sonicated until clear. To prevent overheating the samples, ice was added as needed. The temperature during sonication was kept at least 5 °C above the  $T_m$  of the lipid mixtures.

#### *Liposome size measurement*

Liposome size was measured by photon correlation spectroscopy with a N4+ sub-micron particle size analyzer (Coulter, Miami, FL) [20]. Measurements were done at 24 °C for either BPS or dioleoyl-LBPA/EPC liposomes. Monodispersed LUVs, prepared as described above, were evaluated using the N4+ sub-micron particle size analyzer. Based on preliminary studies showing vesicle fusion within 2–3 min [20], the saposin C-induced liposome fusion was allowed to proceed for 15 min at room temperature. The data for liposome size estimation were acquired at a 90° angle and processed using unimodal and size distribution process (SDP) analysis with a fair autocorrelation function.

#### *R18 (octadecyl rhodamine B) and stopped-flow assays*

Fluorescence probe R18 was entrapped in the internal aqueous compartment of SUVs by co-sonication with phospholipids (lipid:R18 = 96:4, mole:mole). At 8  $\mu$ M, R18 displays self-quenching in vesicles with small sizes. When fusion occurs between non-probed and probed vesicles, the R18 concentration is diluted, resulting in an increase in intensity of fluorescence (dequenching). Therefore, the R18 dequenching assay was used to monitor probed and non-probed vesicle fusion [20]. The dequenching signal was determined by an emission scan with the spectrofluorimeter by exciting the probe at 560 nm. Also, the dequenching signal was continuously measured over time using a stopped-flow apparatus. The stopped-flow experiments were carried out in an SLM-Aminco MilliFlow stopped-flow apparatus [20].

#### *Trp fluorescence spectral analysis*

Singly Trp substituted saposin C (0W) was created by site-directed mutagenesis as described [19,20]. Trp fluorescence emission spectra were acquired in a luminescence spectrometer as described above. Excitation wavelength ( $\lambda_{EX}$  = 280 nm) and spectral bandwidths (4 nm) were used for the excitation and emission monochromators. Trp-saposin Cs (total protein = 2 M) were added to liposome suspensions at protein-to-lipid ratios of 1:20 (mole:mole). The associations between saposins and BPS vesicles were determined by fluorescence emission scan analysis. Liposomal dispersions had no fluorescence under these conditions.

#### *Saposin activation assay and circular dichroism (CD) measurement*

Saposin C's activity toward acid  $\beta$ -glucosidase was determined fluorometrically [9,19,21,22]. Assays were conducted in a detergent-free system with phosphatidylserine liposomes. Ceredase (2 nM) and 4-methylumbelliferyl- $\beta$ -D-glucopyranoside (4 mM) were used for all enzymatic activity assays. The protein-to-lipid ratios were 1:20 (mole/mole). The secondary structures of saposins were estimated from the CD spectra and recorded at room temperature from a Jasco J-710 spectropolarimeter (Jasco, Easton, MD) using a 0.1 cm quartz cell [19,22,23]. To reduce noise at a wavelength below 200 nm, the proteins were dissolved in 10 mM sodium acetate, pH 4.7. These experiments were conducted to predict relative changes in CD spectra.

## Results

#### *Localization of fusogenic domain in saposin C*

To define the regions responsible for the fusion property, recombinant molecules and synthetic peptides containing about 50% of the amino acid sequence of either the amino- or carboxyl-terminal halves of saposin C were tested for induction effect on vesicle size changes. The recombinant molecules with the amino-half of saposin C showed fusogenic activity with BPS liposomes at nanomolar concentrations (Fig. 2A). However, the carboxyl-half of the protein molecule had no effect on the size of BPS vesicles under exactly the same conditions (Fig. 2A). These recombinant proteins contain an extra 14 and 13 amino acids at  $NH_2$ - and  $COOH$ -termini, respectively [9]. To evaluate the possible effects of the non-saposin amino acid sequences, saposins C (6–40) and C (41–80) were synthesized and tested for fusion activity with BPS vesicles. Similar patterns of fusogenicity from synthetic peptides were found with those recombinant saposins (Fig. 2B). Also, saposin B did not induce fusion of the BPS liposomes (Fig. 2B). In our previous studies, synthetic peptides with a single helical region in saposin C, such as saposins C (4–20), C (24–40), or C (67–80), had no fusogenic activity, but showed an inhibitory effect on saposin C-induced lipid vesicle fusion [20]. Based on these results, we concluded that a potent fusogenic domain was located on the amino-terminal half of saposin C, which consists of the first (H-1) and second (H-2) helices. Single helical domains in saposin C were not sufficient to induce liposomal fusion. Also, the five amino acid residues from Ser1 to Cys5 (ISDVYC5) at the amino-terminal of saposin C and the extra amino acid residues from the expression vector sequence had no impact on their fusogenic function. This conclusion was further confirmed by the kinetic study described below.

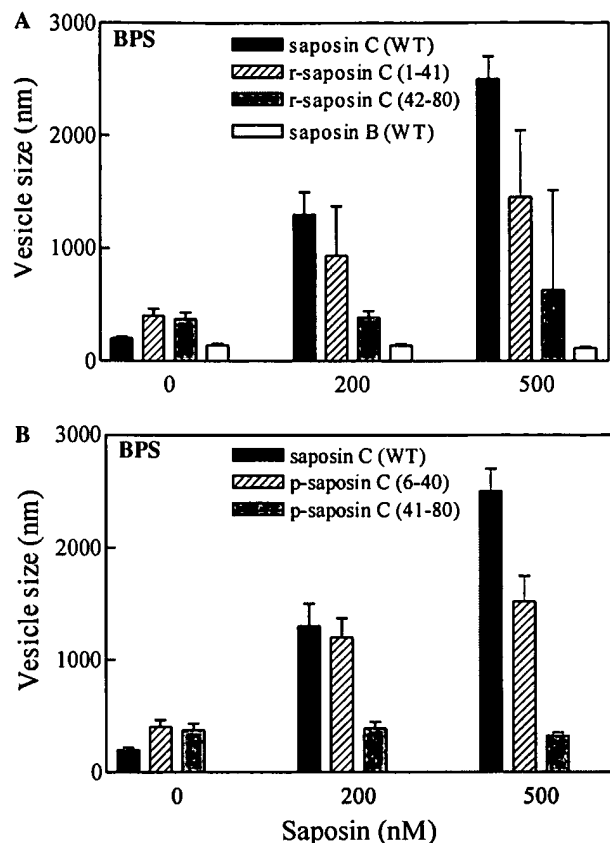


Fig. 2. Size determinations of BPS vesicles in the presence of saposins by N4+ analysis. (A) saposin C (WT): wild-type saposin C; r-saposins C (1–41) and (42–80): recombinant saposin C (1–41) and C (42–80); saposin B (WT): wild-type saposin B. (B) saposin C (WT): and wild-type saposin C; p-saposins C (6–40) and (41–80): synthetic saposin C (6–40) and C (41–80) peptides. Assay conditions: 0.1 M citric/0.2 M phosphate buffer (pH 4.7), room temperature, LUVs were prepared from BPS (20  $\mu$ M). Experiment conditions: room temperature, fair autocorrelation function, base line error <1%, each size represented the mean of a single population of >85% vesicles.

#### Stopped-flow assay

The stopped-flow experiments with R18-containing BPS vesicles were conducted for kinetics and quantitative analysis of minimal fusogenic domain in saposin C (Fig. 3). Such analysis was based on the dequenching of R18 upon dilution while fusion occurs between non-probe and R18-containing liposomes. An increase in R18 fluorescence emission signal was kinetically monitored by using a stopped-flow apparatus as described [20]. Under the acidic environment, R18 dequenching curves were detected when synthetic saposin C (6–40) peptides at 250 and 500 nM were mixed with BPS vesicles at room temperature (Figs. 3A and B). An increase in fluorescence intensity was observed in these time curves. Similar to the control experiment without protein, no increase in fluorescence signal was found in kinetic curves with the saposin C (41–80) peptide.

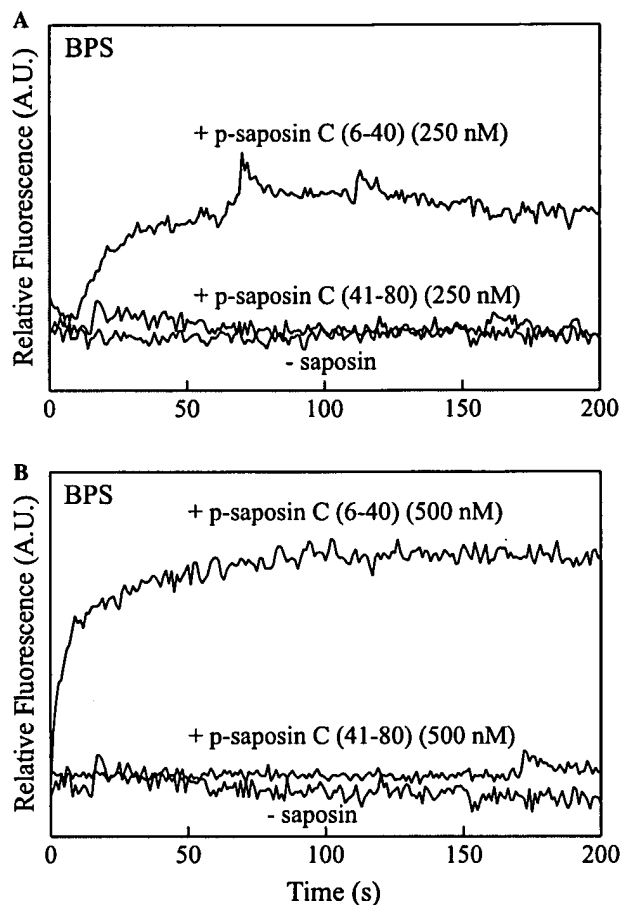


Fig. 3. Stopped-flow analyses of saposin C peptide interaction with BPS SUVs at pH 4.7 (0.1 M citric acid/0.2 M phosphate), room temperature. SUVs entrapped with the fluorescence dye R18 were prepared with BPS. Reaction mixtures: BPS vesicles in the presence or absence of p-saposins C (6–40) and (41–80) (synthetic saposin C (6–40) and C (41–80) peptides). All samples in the left chamber: equal volume of lipid with and without R18; total lipids = 200  $\mu$ M; lipid:R18 = 96:4 (mole:mole). Samples in the right chamber: peptides = 0.5  $\mu$ M in (A) and 1.0  $\mu$ M in (B). Fluorescence assay for R18:  $\lambda_{EX}$  = 560 nm and  $\lambda_{EM}$  = 590 nm.

Again, these data indicated that the amino acid sequence (6E–40P) was a fusogenic domain in saposin C.

#### Localization of fusogenic amino acid residues in saposin C

Increasing evidence supported the notion that negatively charged phospholipids were required for biological functions of saposin C, including membrane fusion induction [15,20]. Thus, the positively charged amino acid residues may play an important role in ionic interaction between saposin C and acid phospholipids. There are a total of seven basic lysine residues in the sequence of saposin C. Interestingly, six of these basic residues reside in the fusogenic domain in the amino-terminal half of the saposin C molecule, except for Lys41 which is located in the non-helical region between

helices 2 and 3 (Fig. 1). This concomitantly suggests a possible role of these lysine residues in saposin C-induced vesicle fusion. All lysine residues in saposin C were individually substituted by alanine using site-directed mutagenesis [21]. Four Lys mutant saposin Cs were obtained. These lysines included Lys13 and Lys17 in helix 1, Lys23 in the non-helical region between helices 1 and 2, and Lys38 in helix 2. After expression in *E. coli*, the mutant saposins C (K13A), C (K17A), C (K23A), and C (K38A) were purified to homogeneity as described [9]. Other lysine mutants at Lys26, Lys34, and Lys41 were not included in the present studies, since these mutant proteins were not able to be produced using the same expression system.

N4+ analyses were conducted with these mutant saposin Cs to determine their effects on size changes of phospholipid liposomes. Under the same experimental conditions, all of the mutant saposin Cs at nanomolar concentrations did not significantly induce size changes of BPS liposomes (Fig. 4A). The majority of in vitro experiments for saposin-lipid interaction were done

with vesicles containing PS [15,19,20,24]. However, the physiological relevance of in vitro functionality studies with saposin C and PS has not been addressed adequately. Most recently, we found the specific interaction of saposin C and LBPA in late endosomes (Qi et al., unpublished data). LBPA is a major phospholipid in late-endosome membranes [25–27]. In vitro, saposin C induces fusion of the LBPA containing vesicle [20]. Therefore, dioleoyl-LBPA was used to prepare liposomal vesicles with EPC by mimicking physiological membranes. Saposin C induced the fusion of the EPC vesicles containing 15% of dioleoyl-LBPA (Fig. 4B). A similar reaction pattern in the presence of the Lys mutant saposin Cs was observed to that with PS containing lipid vesicles in Fig. 4A. These mutants had little or no effect on the sizes of the dioleoyl-LBPA/EPC liposomes.

#### Emission Spectra of R18 dequenching

The above conclusion from N4+ analyses was further confirmed using the core-mixing method with self-dequenching of R18 as a fluorescent probe. Upon addition of wild-type saposin C into the mixture of R18 probed and buffer-filled PS liposomes, an increase in fluorescence intensity in emission spectra was observed due to the fusion of two distinct vesicles (Fig. 5A). In addition, the degree of fluorescence change correlated to the concentration of the protein that was added. To exclude the possibility of saposin C-induced vesicle leakage under similar conditions, the control experiments were conducted in the presence of R18-probed liposomes alone (Fig. 5B). No change of fluorescence emission spectra was observed in the presence and absence of saposin C at 500–800 nM. This indicated that the increases in fluorescence induced by saposin C (Fig. 5A) were not a result of R18 leaking out from the vesicles. Such changes in fluorescence intensity were not observed by addition of the Lys mutant proteins into the mixture of R18-probed and buffer-filled PS vesicles (Figs. 5C–F). Similar results were obtained with dioleoyl-LBPA-containing liposomes (data not shown). These data suggest that the selected lysine residues are critical to the fusogenic function of saposin C.

#### Roles of lysines in enzymatic activation on acid $\beta$ -glucosidase

Saposin C is a physiological activator of a lysosomal hydrolase, acid  $\beta$ -glucosidase (GCase). The enzymatic activation sequence located at the carboxyl-terminal sequence [21] is seemingly segregated from the fusogenic domain in saposin C. In addition, three disulfide bonds and the proper conformation of saposin C are critical to its enzymatic activation function. To determine the roles of Lys residues in the enzymatic activation function, the mutant saposin Cs were used to test for in vitro stimu-

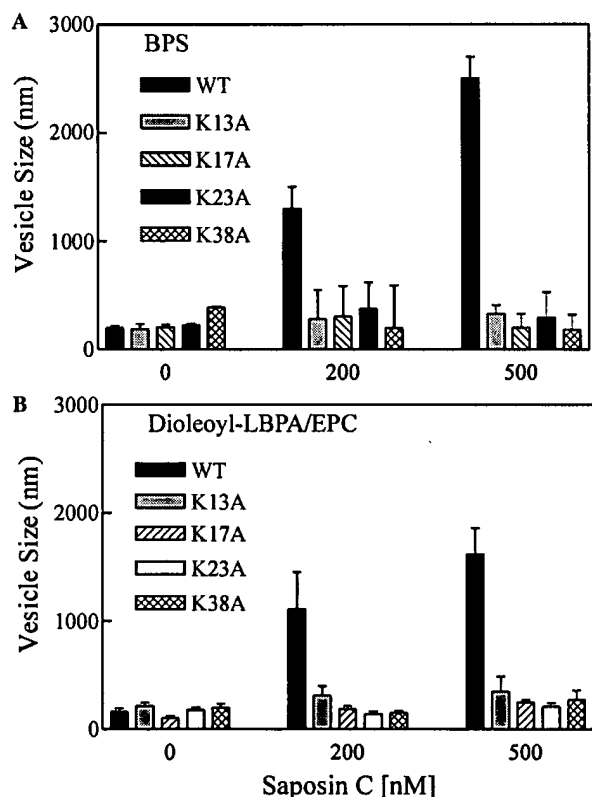


Fig. 4. Size determinations of LUVs in the presence of wild-type and mutant saposin Cs by N4+ analysis. Saposin C (WT): wild-type saposin C; K13A: saposin C (K13A); K17A: saposin C (K17A); K23A: saposin C (K23A); and K38A: saposin C (K38A). LUVs: 20  $\mu$ M of BPS in (A) and dioleoyl-LBPA/EPC (1:6.67, mole:mole) in (B). Assay conditions: 0.1 M citric/phosphate buffer (pH 4.7), room temperature, fair autocorrelation function, base line error <1%, each size represented the mean of a single population of >85% vesicles.

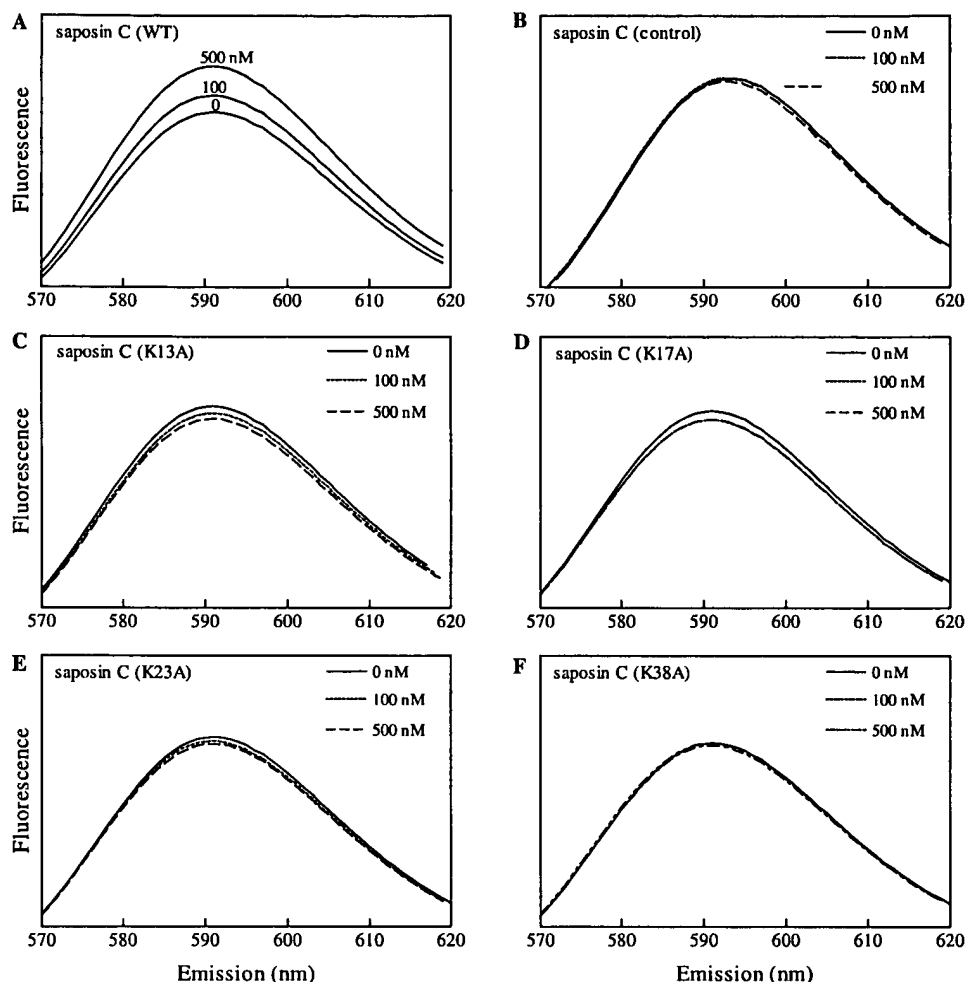


Fig. 5. Emission spectra of R18 containing BPS SUVs in the presence of wild-type (WT) and mutant saposin Cs. The spectra were acquired at  $\lambda_{\text{EX}} = 560$  nm in 0.1 M citric/phosphate buffer, pH 4.7, at room temperature. SUVs entrapped with the fluorescence dye R18 were prepared with BPS. (A and C–F) equal volume of lipid vesicles with and without R18. (B) equal volume of lipid vesicle with R18 and buffer. Final lipid concentration = 100  $\mu\text{M}$ . Total protein concentrations are indicated in the figure. Increasing fluorescence intensity (R18 dequenching) indicated the occurrence of fusion.

lation of GCase activity. Our experiments indicated that saposins C (K13A) and C (K17A) had a similar activation level on GCase as wild-type saposin C (Fig. 6). However, the activation effect of other mutants, C (K23A) and C (K38A), was dramatically diminished at a concentration up to 480 nM. Based on these results, two Lys residues (K13 and K17) in saposin C were critical for fusogenic activation, but not to the enzymatic stimulation function.

#### Effects of lysines on saposin C conformation

Single mutated saposins C (K23A) and C (K38A) had neither the fusogenic function on liposomal vesicles nor the activation effect on GCase. In addition to disruption of the electrostatic interaction between the positive charged residues and the negatively charged membrane surface, other potential alterations of saposin C may have an impact on the GCase activation by these two

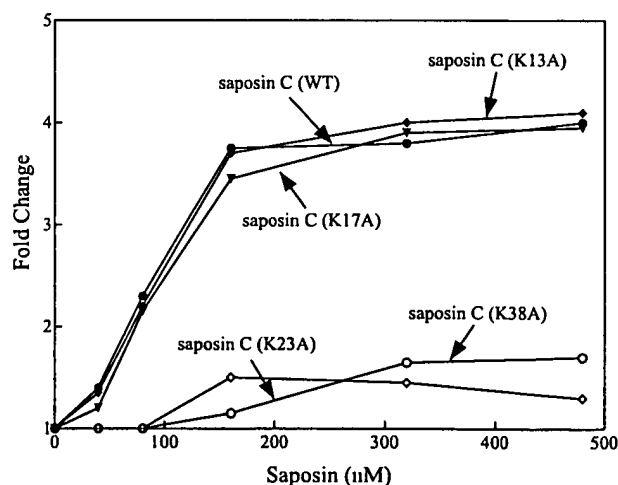


Fig. 6. Activation profiles for GCase by wild-type and mutant saposin Cs. All assays were conducted with 2 nM GCase and 400 ng/ml BPS liposomes in 0.1 M citric/0.2 M phosphate (pH 4.7).

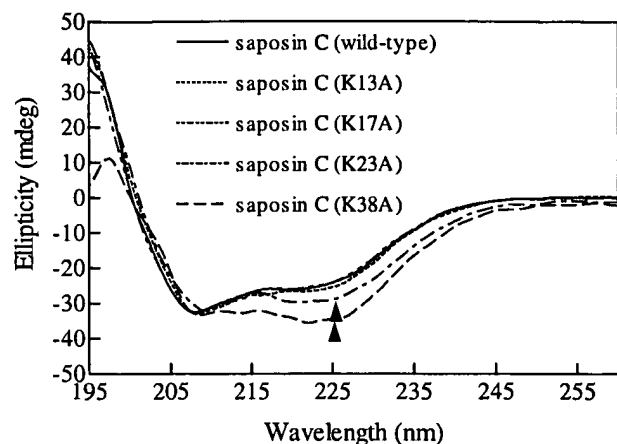


Fig. 7. CD spectra of wild-type and mutant saposin Cs. Spectra were acquired in 0.005 M sodium citrate/0.01 M sodium phosphate (pH 4.7) at room temperature with 0.2 mg/ml saposin Cs.

mutations. The relative secondary structures of the Lys mutant proteins were estimated by circular dichroism (CD) spectroscopy (Fig. 7). The CD spectra changes of saposins C (K23A) and C (K38A) in a region around 225 nm were obtained in comparison to wild-type saposin C (see arrowheads in Fig. 7). The superimposed CD curves between wild-type and mutant saposins C (K13A) and C (K17A) indicated that these two mutated proteins preserved the normal conformation of saposin C. These experiments showed that the alterations of saposin C conformation by substitution of Lys23/Lys38 with alanine affected the stimulation action on GCase, as well as fusogenic activity. Taken together, these results also suggest that basic amino acids (at least Lys13 and Lys17) at the amino-terminal half of saposin C are critical to its fusogenic activity, but not necessary for its activation function on GCase.

#### Effect of lysines on membrane insertion of saposin C

To evaluate the membrane insertion properties of the Lys mutant saposin Cs, fluorescence emission shift analysis was conducted as described [19]. Since saposin C is a Trp-free protein, a Trp was added as an intrinsic fluorescence probe before the first amino acid residue in saposin C, named as saposin C (0W). Such addition of Trp did not alter the fusogenic effect and enzymatic activation of saposin C [19,20]. Therefore, Trp-saposin Cs were created with the backbone of the Lys mutated saposin Cs, named as saposins C (0W/K13A), C (0W/K17A), C (0W/K23A), and C (0W/K38A). A blue shift of Trp fluorescence emission in saposin C (0W) in the presence of BPS liposomes was observed, due to the polarity change of the tryptophan environment after the protein insertion into the membrane [19]. Similar blue shifts were found with all the Lys mutated saposin C (0W) upon the addition to the suspension of BPS

Table 1

Fluorescence emission maxima of Trp-saposins in the absence and presence of BPS

Saposins	Emission maxima (EM, nm)		EM shift
	–BPS	+BPS	
C (0W)	339	333	Blue
C (0W/K13A)	346	333	Blue
C (0W/K17A)	346	334	Blue
C (0W/K23A)	346	335	Blue
C (0W/K38A)	345	332	Blue

Experiments were conducted in 0.1 M citric acid/0.2 M phosphate (pH 4.7), at room temperature. Total protein concentration was 2  $\mu$ M. The protein:lipid ratio was 1:20 (mole:mole). The excitation wavelength was at 280 nm.

vesicles (Table 1). These data indicate that individual mutation of lysines at positions 13, 17, 23, and 38 did not prevent saposin C from embedding into the BPS liposomal membrane.

#### Discussion

The fusogenic domain in saposin C was localized at the amino-terminal half (amino acid residues 6–40) of the molecule in the present experiments. This domain contains the first and second helices, namely, H-1 and H-2 (Fig. 1). It suggests that both of the helical peptides contribute to fusion processing. In fact, a single individual helix in saposin C was not sufficient to induce liposomal membrane fusion. According to the proposed clip-on model as previously described [20], the H-1 region is responsible for membrane insertion, while H-2 is required for inter-saposin interaction to bring the opposite vesicles together during saposin C-induced membrane fusion. Our present studies provide additional evidence to support the clip-on model. These results indicate the segregation of the fusogenic domain and the activation region in saposin C, since the sequence at the NH<sub>3</sub>-terminal half is not required for saposin C's activation on GCase [21]. In addition, mass spectrometry analyses showed that the synthetic peptides, saposins C (6–40) and C (41–80), were monomeric forms. However, some of the recombinant saposins C (1–41) and C (42–80) were dimers linked by inter-molecular disulfide bonds [21]. In the present study, fusion patterns of the synthetic peptides were similar to those of the recombinant saposin Cs. This suggests that the fusogenic function of saposin Cs is not significantly affected by the inter-molecular disulfides.

Although the (His)<sub>6</sub> sequence was tagged on the carboxyl terminus of the recombinant saposin C, the previous experiments indicated that the His tag had no significant impacts on the enzymatic activation and membrane insertion activity of saposin C [9,19]. However, there is a possibility that the (His)<sub>6</sub> tail might have

an influence on the fusogenic activity of saposin C. Therefore, the synthetic saposin C peptides without (His)<sub>6</sub> sequence were used to compare the membrane fusion induction with the recombinant saposin Cs. In the present assay systems, similar fusogenic patterns were found from the synthetic saposin peptides without the tag and the recombinant saposin Cs with the tag. Thus, the impact of the His tag on the fusogenic activity of saposin C, if any, should be minor.

Our previous studies showed that the H-1 and H-5 helices in saposin C inserted partially into the lipid membrane [19]. Based on the computational calculations of the hydrophobic and hydrophilic centroids of individual helices (Liu and Qi, unpublished data, [28]), the overall hydrophobic and hydrophilic centroids of these helices are located at their amino- and carboxyl-terminal parts, respectively. Therefore, the amino-terminal part of H-1 with more hydrophobic property is preferably embedded into the membrane. The carboxyl terminal of H-5 with more hydrophilic property does not prefer to be embedded into the lipid membrane. However, H-1 and H-5 are bound by two disulfide bonds. The hydrophobic amino-terminal of H-1 and the hydrophilic carboxyl terminal of H-5 were observed to be inserted into the membrane during saposin C-induced membrane fusion. Thus, H-5 may provide an additional stiffness and stability for membrane insertion of the intact saposin C.

Growing evidence showed that saposin C binds negatively charged, unsaturated phospholipids [15,19,20, 24]. These experiments suggest an initial electrostatic interaction between positively charged residues in saposin C and the negatively charged membrane surface. Interestingly, most of the basic amino acid residues (six out of total seven lysines) are located in the fusogenic domain of saposin C (Fig. 1). Saposin B, another saposin family member, had no fusion effect on BPS vesicles (Fig. 2A). This saposin contains three lysine residues (Fig. 1), but only one is located in the region that corresponds to the fusogenic domain in saposin C. The lysines appear to be critical to the ionic interaction between saposin C and the acidic lipid membrane. By taking the site-directed mutagenesis approach, the individual replacement of the lysines (Lys13, 17, 23, and 38) with alanine in the fusogenic domain of saposin C resulted in a complete loss of fusion induction on the BPS or LBPA/EPC liposomes. These results indicated that these lysines were required for the fusogenic function of saposin C. In addition to the basic residues, saposin C has 16 acidic residues, i.e., glutamic and aspartic acids. The net charge of saposin C should be negative at neutral pH. At the fusogenic pH (acidic pH), the  $\beta$ - and  $\gamma$ -carboxyl groups of Asp and Glu are not fully ionized since their  $pK_R$  values are around 4. However, the  $\epsilon$ -amino group ( $pK_R = 10.5$ ) of lysine in saposin C has a positive charge. In this case, total negative charges were reduced,

and the ratio of positive/negative charges is increased. Therefore, saposin C-induced fusion was favored in vesicles containing negatively charged phospholipids in a low pH environment.

An additional functional assessment for the Lys mutations is to test the *in vitro* enzymatic activation on GCase. The K13A and K17A mutants had the same levels of activation in comparing to the wild type protein. But saposins C (K23A) and (K38A) showed no effect on the GCase activity (Fig. 6). The secondary structural changes were found with K23A and K38A, but not with K13A and K17A by circular dichroism (CD) (Fig. 7). These data indicated that the K13 and K17 residues were critical to the fusogenic function of saposin C, but not for the enzymatic activation on GCase and its secondary structure. The K23A and K38A mutants altered the enzymatic activation and fusogenic activities, due to conformational changes of saposin C. The role of the positive charges of Lys23 and Lys38 in saposin C-induced fusion needs further investigation by using different mutations which have no impact on the conformation of saposin C.

The effect of Lys mutations on the membrane insertion of saposin C was evaluated by fluorescence emission shift analysis. Trp in saposin C (0W) was used as the fluorescence membrane-embedding probe for emission scan assays [19]. Blue shifts of maximum fluorescence emission were observed from wild-type and mutant saposin Cs upon mixing with BPS vesicles (Fig. 7). These results suggest that the tested individual lysines are not critical for the membrane insertion of saposin C. Usually, two different types of protein–lipid–water forces, electrostatic and hydrophobic, have been considered for protein–lipid membrane interactions. A single lysine mutation may not be sufficient to ablate the initial ionic interaction between saposin C and the negatively charged membrane. Consequently, the overall amphipathic property of H-1 in saposin C ensures the membrane insertion. Lys13 and Lys17 mutants lost the fusion-induction activity and retained the enzymatic activation, albeit the membrane insertion occurred. It suggests that the fusogenic activity of saposin C is strictly based on the manner of membrane anchoring. Such fusion-inducing processing by saposin C, perhaps, depends on the proper orientation and insertion angle of helices in the lipid membrane. Lysine residues, at least Lys13 and Lys17, are necessary to maintain the detailed helical peptide–membrane association. For example, Lys13 and Lys17 may be important to stabilize the helix 2 structure that is required for saposin C-induced membrane fusion.

In summary, the fusogenic domain was located at the NH<sub>3</sub>-terminal half of saposin C, although the first five amino acid residues were not required. In addition, a linear sequence consisting of H-1 and H-2 in saposin C is sufficient to induce the BPS membrane fusion.



Basic amino acids, Lys13 and Lys17, were critical for the fusogenic activity of saposin C, but not required for the activation of GCase. The segregation of fusion-inducing and enzymatic activation functions provides a base for investigating the physiological relevance of saposin C's fusogenicity in cells and animal models.

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